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TECHNICAL MANUSCRIPT 410

ETHYLENE OXIDE STERILIZATION RATES AND PROTECTIVE INFLUENCES

Robert K. Hoffman

AUGUST 1967

DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

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TECHNICAL MANUSCRIPT 410

ETHYLENE OXIDE STERILIZATION RATES AND PROTECTIVE INFLUENCES

Robert K. Hoffman

Physical Defense Division
COMMODITY DEVELOPMENT AND ENGINEERING LABORATORY

Project 1B622401A072

August 1967

ETHYLENE OXIDE STERILIZATION RATES AND PROTECTIVE INFLUENCES

ABSTRACT

A review of the literature on ethylene oxide sterilization is presented. This review shows that the death rate of microorganisms exposed to ethylene oxide is dependent on such variables as oxide concentration, relative humidity, temperature, and the type of microorganism involved plus the protective influences of its surrounding medium. In many respects, ethylene oxide resembles heat, whose sterilizing effect also depends on concentration (temperature), relative humidity, and the specific microorganism and its surrounding medium. Heat, however, penetrates more readily and sterilizes material interiors, while ethylene oxide's ability to penetrate varies with the material involved. Thus, ethylene oxide is recommended primarily as a surface sterilant.

Ethylene oxide is the most widely used of the chemical sterilizing gases. It is a low-molecular-weight, three-membered, cyclic compound having the structure shown in the Figure 1. It boils at 10.8 C, so it is a gas at room temperature when unconfined. It is a non-corrosive chemical in the gaseous state, but liquid ethylene oxide is a solvent for some plastics. It kills all forms of microorganisms, including resistant soil organisms. Its high penetrating power and non-corrosiveness make it an ideal sterilant for delicate electronic equipment and numerous other heat-labile items. Ethylene oxide is not only a sterilant for surfaces; it readily penetrates and sterilizes porous materials, such as textiles or paper and some thin plastics and other organic solids in which it can dissolve. However, it will not penetrate hermetically sealed areas or sterilize subsurface as do heat and penetrating radiation.

Little has been published on the rate of microbial inactivation by ethylene oxide gas. The first paper from our laboratories, by Phillips[1] in 1949, covered the effects of concentration (up to 884 mg/liter), temperature (up to 37 C), and time upon ethylene oxide sterilization. The effect of relative humidity was covered in a companion paper (Kaye and Phillips[2]), and RH efrects were reinvestigated by Gilbert, Gambill, Spiner, Hoffman, and Phillips[3] in 1964. In 1962 Ernst and Shull[4] extended the Phillips' data to higher temperature (55 C) and concentration (1,500 mg/liter) ranges. Oth publications deal primarily with ethylene oxide's ability to sterilize various materials and disregard death rate determinations.

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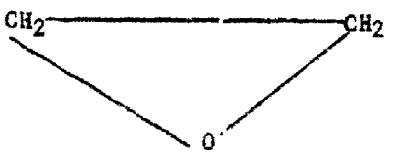

Figure 1. Chemical Structure of Ethylene Oxide.

The death rate of microorganisms exposed to ethylene oxide gas not only is dependent on temperature, relative humidity, and gas concentration, but also on the species of microorganism, including its surrounding menstruum and past history. The effect of relative humidity on the ethylene oxide inactivation rate is probably the most complicated, as is well demonstrated by Gilbert.[3] His results for B. subtilis var. niger spores exposed on cloth patches at 25 C to 120 mg of ethylene oxide per liter at various relative humidities are shown in the next two figures.

In these tests, the patches were contaminated with a measured quantity of B. subtilis var. niger spores in aqueous suspension and dried at <1, 11, 22, 33, 53, 75, or 98% relative humidity maintained by a dry salt or various saturated salt solutions. After equilibrating one or more days, the contaminated patches were rapidly transferred to another desiccator for ethylene oxide exposure. Here the humidity was quickly adjusted to the same level as that at which the patches were dried. Following this, a slight vacuum was pulled (pressure reduced from about 750 to 700 mm Hg), and the pressure was then returned to atmospheric with ethylene oxide gas. This gave a concentration of 120 mg of the oxide per liter of space.

Figure 2 shows the death rates for spores equilibrated and tested at 33, 53, 75, and 98% relative humidity. The D values for these curves are 0.8, 1.3, 2.1, and 1.9 hours, respectively. It is apparent that spores on cloth were killed more slowly as the relative humidity increased from 33 to 98% RH, but, even so, straight line death rates were obtained over the entire range.

Figure 3 shows the death rates for spores conditioned to relative humidities of 1, 11, 22, and 33% and then exposed to ethylene oxide at the same humidities. Here, the death rates for the spores tested at humidities below 33% are not linear like those for higher RH's, so no D value can be calculated to describe the complete death rate. This emphasizes the importance of moisture when using ethylene oxide. Granted, 99.9 to 99.99% of the spores were readily killed at these low relative humidities, but sterility was not attained even after an ethylene oxide exposure of three days. The failure to achieve sterilization cannot be attributed to a low gas concentration because increasing it from 120 to 950 mg per liter did not sterilize the desiccated organisms. The results obtained with B. subtilis

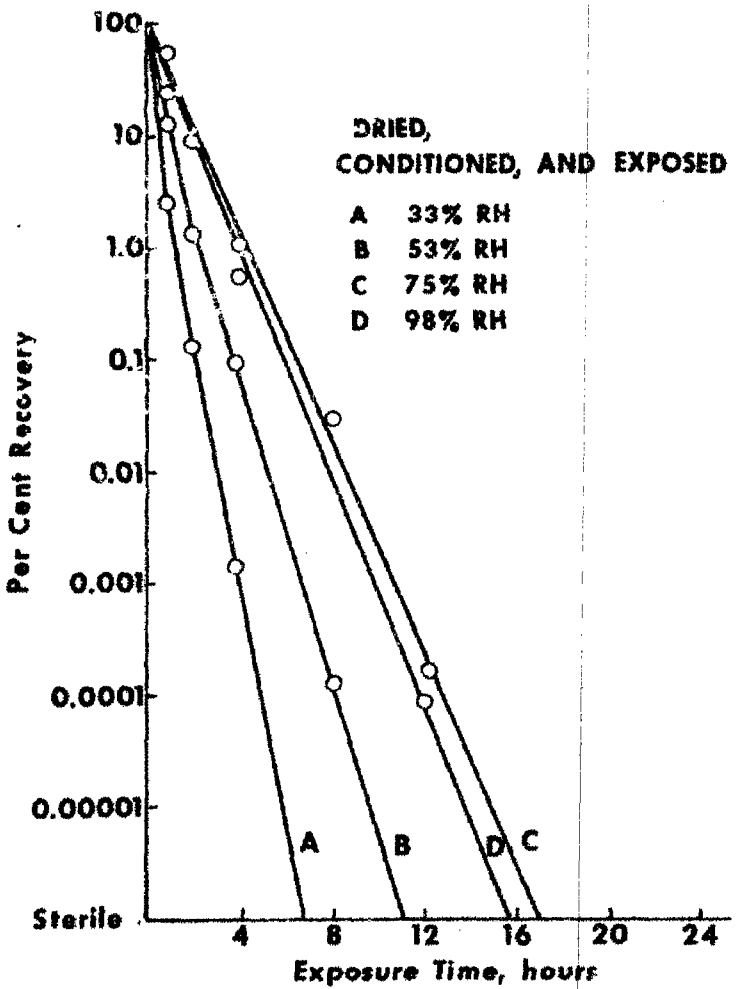

Figure 2. B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide 120 mg/liter at 25 C.

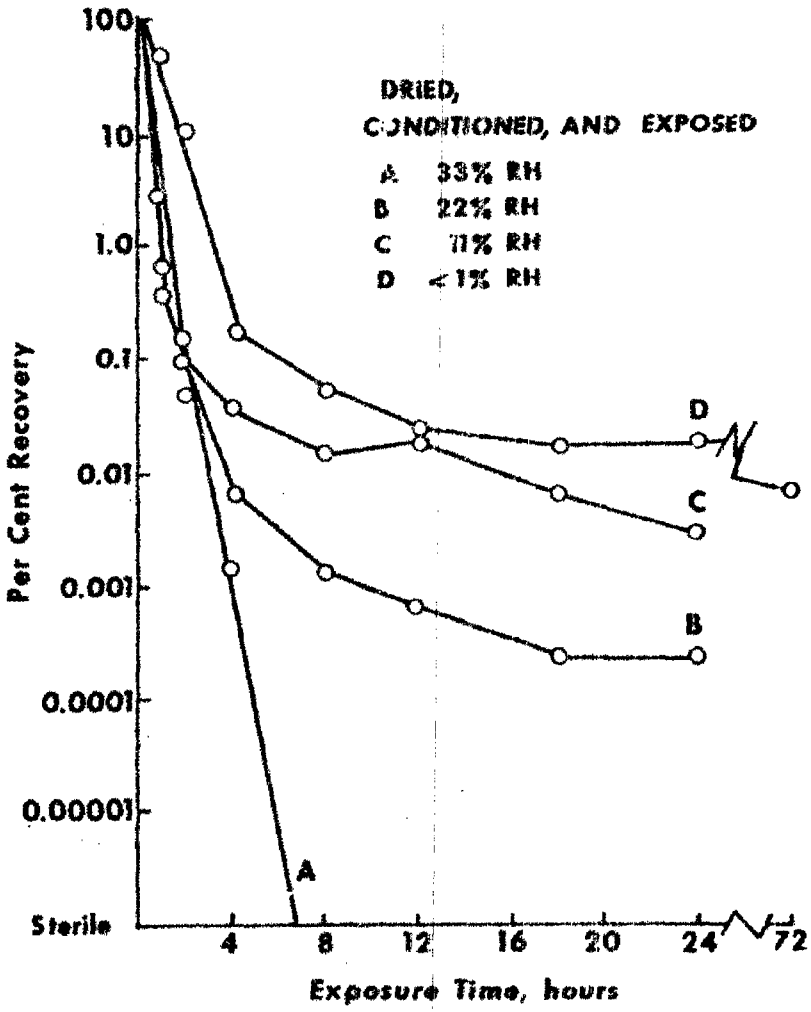

Figure 3. B. subtilis Spores on Cotton Patches Exposed to Ethylene Oxide 120 mg/liter at 25 C.

var. niger spores on porous surfaces, such as cloth or filter paper, reveal that the fastest death rate occurs at a humidity around 33%. The need for a higher relative humidity to sterilize impervious surfaces such as glass has been pointed out by Kaye and Phillips, Gilbert et al., and Ernst and Shull.[3-4] To insure sterility of those surfaces, the humidity must be increased to at least 40%.

In 1949 Kaye and Phillips pointed out that ethylene oxide becomes a better disinfectant the drier the air and material being treated becomes, but they indicated this is true only up to a point where, if almost all the moisture is removed, the phenomenon is sharply reversed.

The roll that moisture plays in the ethylene oxide inactivation of the microbial cell is still not clearly understood. Kaye and Phillips, Mayr,[5] and Shull[6] all suggested that the bacterial cell surface might control the penetration of ethylene oxide, and that it is only when this surface is moist that the oxide can be absorbed by the cell. A second theory suggested by Kaye and Phillips was that moisture might be required to assist the ionization of sulfhydryl, amino, carboxyl, or hydroxyl groups in the cell for alkylation by ethylene oxide. Both of these explanations now seem unlikely because later data from our laboratory[3] showed that most organisms are killed as readily in a desiccated ethylene oxide atmosphere as in a non-desiccated atmosphere. It is only about 0.1% or less of the organisms that are resistant to ethylene oxide sterilization in a desiccated atmosphere.

D values for the initial portions of Gilbert's death rate curves at low RH can be calculated because approximately straight line relationships exist for up to 99.9% kill. The D values thus obtained are 0.8, 0.8, and 1.5 hours, respectively, for 22, 11, and 1% RH, which are lower than the 2-hour value obtained for 75 and 98% RH.

These results show that little free water is required in the cell for the ethylene oxide inactivation reaction. Gilbert et al. suggested that cross-linkage occurs at a critical site in a small percentage of cells subjected to desiccation, thus decreasing its availability for alkylation by ethylene oxide. When the cell is rehydrated, the cross-linkage is ruptured, and the cell is once again susceptible to ethylene oxide sterilization. Even if this is correct, the fact remains that resistance appears in only a very small percentage of the cells.

A desiccated, resistant state can develop in subtle ways when treating materials with ethylene oxide. For example, the desiccation effect was especially noticeable when we treated contaminated materials contained in plastic bags with ethylene oxide. The air and its moisture are forced out of the bag to make room for the mixture of ethylene oxide and Freon to expand, thus avoiding over-pressurizing and rupturing the fragile bag. When materials containing sufficient moisture to rehumidify the air are not

present in the bag, sterilization is not achieved, although a kill of up to 99.9% is usually obtained. A similar effect can occur in a rigid chamber when an ethylene oxide mixture replaces air and its moisture. Again, unless water is added, sterilization will not be achieved. Moisture can be supplied by (i) adding water vapor to the ethylene oxide mixture as it enters the chamber, (ii) placing a moist sponge or towel in the chamber, or (iii) including material with sufficient natural moisture to raise the atmospheric humidity. The latter two techniques are applicable to smaller chambers.

Phillips determined the CD (concentration x decimal reduction time) values for five ethylene oxide concentrations ranging from 22 to 884 mg/liter. He concluded that the CD value for any one temperature between 5 and 37 C was close to a constant, so the coefficient of dilution is approximately unity. This means that, if the concentration of ethylene oxide is doubled, the time required to sterilize is halved. Ernst and Shull disagree with this, stating that the CD values increase with an increase in ethylene oxide concentration. Close inspection of their data shows that, in general, the CD does vary with ethylene oxide concentration, but they fail to point out that the variation is also temperature-dependent below 40 C. Ernst and Shull determined the death times for three concentrations of ethylene oxide (440, 880 and 1500 mg/liter) over a temperature range of 20 to 55 C. When the ratio of the CD value for 1500 mg/liter to the CD value for 440 mg/liter is plotted against temperature, the curve shown in Figure 4 is obtained.

The dependence of the CD ratio on temperature is evident by the straight line function, which appears to deviate only when the temperature exceeds 35 to 40 C. The higher the temperature, at least up to about 37 C, the greater the ratio variation. At temperatures of 40 to 55 C the ratio is not temperature-dependent. At 21 C (room temperature), however, the ratio is one; in other words, the coefficient of dilution is unity. When the ratios of the CD values for 880 and 440 mg/liter are plotted against temperature, the B curve on this graph is obtained. This curve shows even less deviation from unity, and, furthermore, the deviation starts at temperatures above 25 C. Again, the ratio is independent of temperature between 40 and 55 C.

We use the unity value as a rule of thumb in our laboratory. This is warranted because we employ low concentrations of ethylene oxide (300 to 650 mg/liter) at room temperature rather than at elevated temperatures. Using ambient temperature also eliminates the need for complicated and expensive temperature control chambers in which to treat materials.

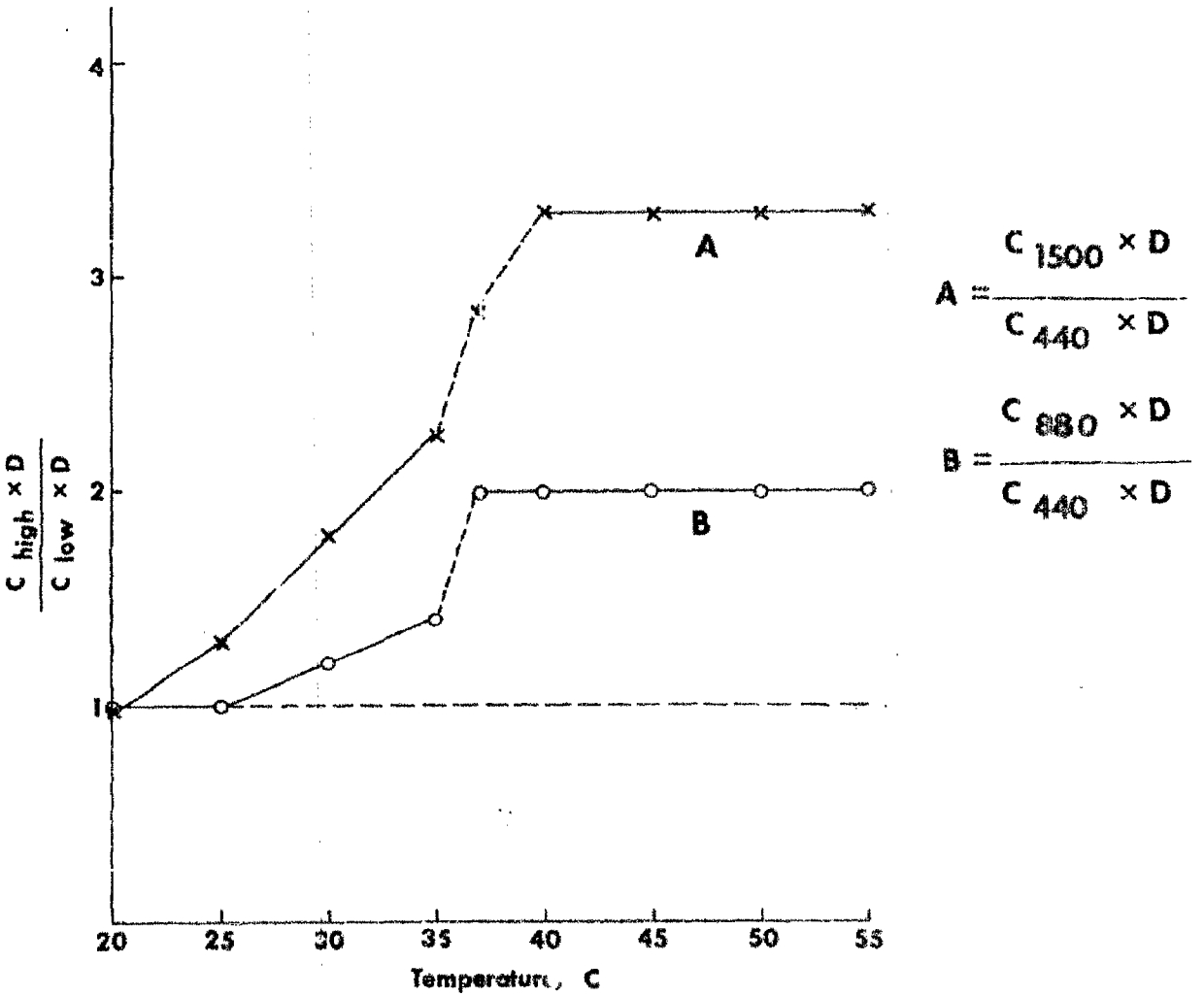

Figure 4. Ratio of Ernst and Shull's High-Low CD Values as a Function of Temperature.

Phillips also determined from his somewhat limited data that there was one temperature coefficient or Q_{10} (2.7) for ethylene oxide used at temperatures from 5 to 37 C. This means that, for each ten-degree increase in temperature, the effectiveness of the oxide increases by a factor of 2.7. On the other hand, Ernst and Shull claim that each concentration of ethylene oxide has at least two temperature coefficients, except when a high oxide concentration (e.g., 1500 mg/liter) is used. At this point, a single Q_{10} of about 1.8 is obtained for temperatures ranging from 25 to 55 C. The data of Phillips and Ernst and Shull are shown in Table 1.

TABLE 1. TEMPERATURE COEFFICIENTS (Q_{10}) AS A FUNCTION OF ETHYLENE OXIDE CONCENTRATION AND TEMPERATURE

Investigator	Concentration	Temperature Range, C	Q_{10}
Phillips	22-884	5-37	2.7
Ernst	440	20-40	3.1
		40-55	1.8
	880	20-35	2.5
		35-55	1.8
	1500	20-55	1.8

Actually, the data of Phillips and Ernst and Shull agree quite well in the areas where they overlap. The average temperature coefficient obtained by each investigator for lower ethylene oxide concentrations and ambient temperatures is 2.7 to 2.8.

The next two figures show some of the equipment that we have used as ethylene oxide chambers. Figure 5 shows a polyethylene bag measuring 2½ by 6 feet. The plastic is 4 mils thick. Materials to be sterilized are placed in the bag, along with a can of ethylene oxide mixture, and the top of the bag is twisted closed and tied. The ethylene oxide can is held from the outside of the plastic, and the valve is opened to initiate sterilization.

Figure 6 shows a rigid, ½-inch-thick, Lucite chamber used to investigate the internal microbial contamination of electronic components. I believe we were the first to demonstrate the presence of living microorganisms

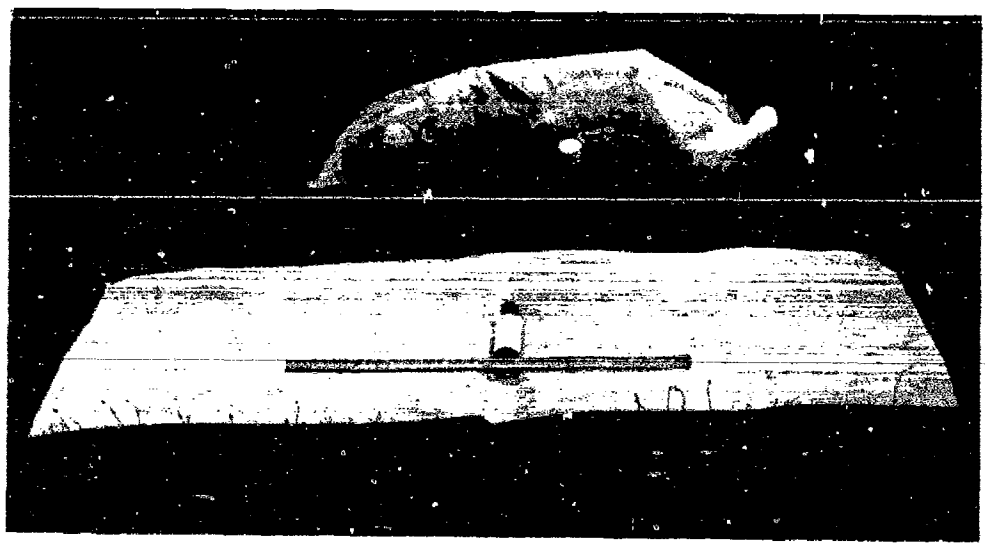

Figure 5. Polyethylene Bag Used in the Ethylene Oxide Sterilization of Small Items.

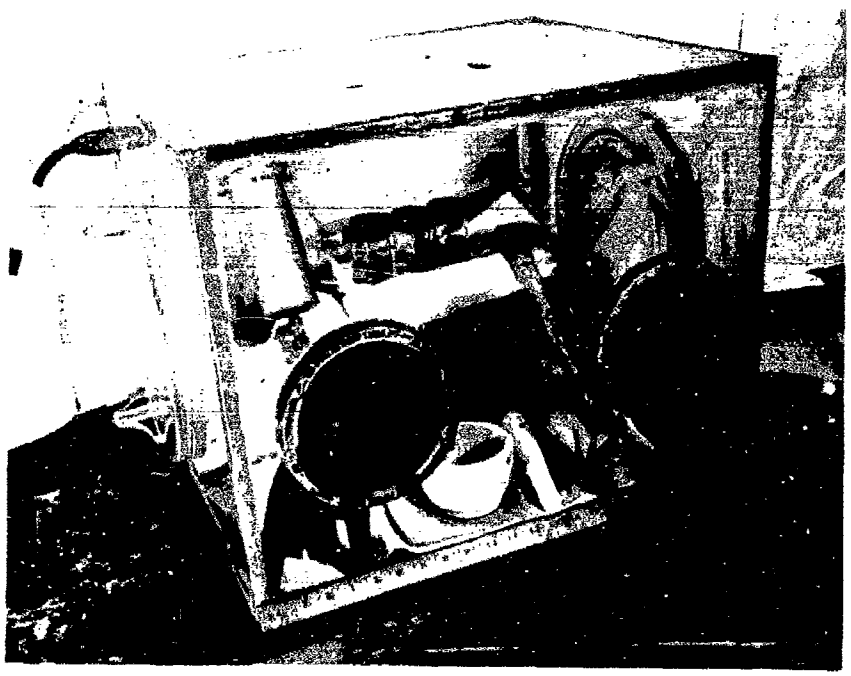

Figure 6. Airtight Lucite Chamber Used to Study the Microbial Contamination of Electronic Components.

insids of hermetically sealed electronic components. All components investigated were placed in this chamber, and their exteriors and the chamber air were sterilized by a six-hour exposure to ethylene oxide at room temperature. Following overnight aeration, the components were cracked open, pulverized as well as possible, and placed in broth medium to check for bacterial growth.

We highly recommend that a moist cloth be placed in both the bag and the chamber, when treating materials with ethylene oxide, to assure sterilization.

It is undoubtedly safe to assume that all materials in which microorganisms are embedded protect the organisms to some degree from the lethal effect of ethylene oxide. In most cases, this protection probably results from the reduced diffusion of the oxide and water in the material rather than from any chemical reaction between the material and the oxide. Because the diffusion rate is considerably slower, vacuum is often used to speed the diffusion of ethylene oxide gas. As noted previously, moisture must be added after evacuation to give a sufficiently high humidity to assure sterilization.

Phillips* tested the effect of ethylene oxide on bacterial spores suspended in oil and water. These studies showed that ethylene oxide gas will dissolve in and diffuse through the media to kill microorganisms suspended therein. Both liquids act as protective materials because they increase the exposure time required for sterilization. The time required to sterilize is a function of depth. This is shown in Table 2. In these tests, Phillips used various depths of olive oil and water, containing B. subtilis var. niger spores, in test tubes. These were exposed for 24 hours in a chamber containing 450 mg ethylene oxide per liter of space at room temperature. The olive oil was sterilized to a depth of 1 cm, but, as the oil depth increased, less and less kill was obtained. Water was sterilized to a depth of 1.8 cm, and less kill was obtained as depth increased.

* C.R. Phillips, personal communication.

TABLE 2. PER CENT KILL OF B. SUBTILIS VAR. NIGER SPORES IN OLIVE OIL AND WATER EXPOSED TO 450 MG/LITER OF ETHYLENE OXIDE FOR 24 HOURS AT 25 C

Liquid		Per Cent Kill	
Depth, cm.	Volume, cc	Olive Oil	Water
0.8	1	100	100
1.8	2½	98	100
3.5	5	91	88
6.8	10	57	55

Allison[7] showed the protective influence of soil on microorganisms exposed to ethylene oxide. In these experiments, the chamber with the soil samples was first evacuated to assist the penetration of ethylene oxide. Allison claims the oxide penetration varies with the soil type, and, for this reason, aggregated soils were sterilized to depths of 5 to 10 cm, whereas finely divided soils, especially silt loams, had to be spread out in layers not more than 1-cm deep to be sterilized. He made no effort, however, to control the moisture content of the soil during the ethylene oxide treatment. In contrast, Mayr showed that bacterial spores in soil are killed more readily at higher relative humidities. Under the circumstances a high relative humidity is probably required to satisfy the moisture requirements of the soil and humidify the spores as well. It would appear that the major problem in attempting to sterilize dry materials is not so much the penetration of ethylene oxide as it is the penetration of moisture.

Dick and Feazel[8] showed that plastics have variable resistance to ethylene oxide penetration. For example, Cellophane 300 PT 62 and polyvinyl alcohol film have very low permeabilities. Polyethylene is somewhat more permeable, polyvinyl chloride considerably more permeable, and ethylcellulose film even more permeable.

This variation in the permeability of plastics has been put to good use in Royce's Sachets, manufactured by Boots Pure Drug Co., as an ethylene oxide sterilization indicator. The Sachet is a small plastic bag containing acidified magnesium chloride solution plus a dye. The thickness of the plastic in the bag is such that a color change occurs only after exposure to an ethylene oxide concentration-time product of 3800 mg hours per liter at 20 C. In other words, if a concentration of 380 mg of ethylene oxide per liter is used in the chamber, it will require 10 hours exposure at 20 C before sufficient oxide diffuses through the plastic to cause a color change.

Recently tests were performed in our laboratory to determine the effect of plastic thickness on the rate of kill of bacterial spores within. Polyethylene plastic, 2 mils thick, was used. In these tests, small cloth patches were contaminated with a measured volume of B. subtilis var. niger spore suspension and then dried at 53% relative humidity at 25 C. After 24 hours, one contaminated patch was placed in each of a number of small bags of polyethylene, and each bag was heat-sealed. Some bags were used singly; others were placed inside one, two, or three more bags of the same material with each bag heat-sealed. Thus, the ethylene oxide had to diffuse through 2 to 8 mils of polyethylene, depending on the number of bags, to react with the bacterial spores on the cloth. The plastic bags containing the patches were placed in a 10-liter desiccator, which was then partially evacuated and returned to atmospheric pressure with the admission of ethylene oxide. A concentration of 650 mg of ethylene oxide per liter of space was used. After various exposure times at 25 C, the patches were assayed for viable spores.

As Table 3 shows, sterility was not obtained in 4 hours regardless of the number of bags used in the first test. In fact, it appeared that the thickness of polyethylene made (i) a considerable difference in the percentage kill in the 1-hour exposure trial (ii) less difference in the 2-hour exposure, and (iii) essentially no difference in the 4-hour exposure.

As stated previously, the patches were pre-equilibrated to 53% RH, a sufficiently high RH to afford complete kill of the spores by ethylene oxide. However, these tests were run during the winter when the ambient laboratory RH was low. The loss of sufficient water during transfer of the patch from the pre-equilibration chamber to the bag and heat-sealing was suspect, so further tests were conducted in which a moist patch was placed in each bag along with the contaminated patch. The results obtained are in the lower part of Table 3. Sterility was reached in 4 hours regardless of the number of bags.

All organic materials discussed so far can be sterilized with ethylene oxide if treated in thin layers. In contrast, even small amounts of some materials, such as crystals of inorganic and organic salts, provide complete protection to microorganisms from the lethal action of ethylene oxide. This has been shown by a number of investigators. For example, Abbott et al.[9] showed that Rochelle salt crystals grown from contaminated liquor could not be sterilized, even with a 96-hour exposure to ethylene oxide. Likewise, Royce and Bowler[10] and Doyle and Ernst[11] showed ethylene oxide's inability to sterilize contaminated glucose, calcium carbonate, sodium chloride, and glycine crystals. The obvious reason for this is that ethylene oxide reacts readily with these chemicals, thus becoming unavailable for penetration and killing microorganisms within.

TABLE 3. STERILIZATION OF B. SUBTILIS VAR. NIGER SPORES IN POLYETHYLENE BAGS EXPOSED TO 650 MG/LITER OF ETHYLENE OXIDE AT 25 C

Moist Patch in Bag	Exposure Time, hours	% Recovery from Indicated Number of 2-mil Polyethylene Bags			
		1	2	3	4
No	1	0.11	0.49	2.8	8.9
No	2	0.0074	0.023	0.058	0.12
No	4	0.001	0.0007	0.0013	0.0038
Yes	1	-	-	-	-
Yes	2	0.0002	0.0014	0.04	0.21
Yes	4	0.0	0.0	0.0	0.0

Thus, the rate at which microorganisms are killed by ethylene oxide depends on such variables as oxide concentration, relative humidity, and the type of microorganisms and their surrounding medium. In many respects, ethylene oxide resembles heat, whose sterilizing effect also is dependent on concentration (temperature), relative humidity, and the microorganism and its surrounding medium. Heat, however, penetrates more readily and sterilizes material interiors, while ethylene oxide's ability to penetrate varies with the material involved. Therefore, ethylene oxide is recommended primarily as a surface sterilant.

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14. Key Words
*Ethylene oxide
*Sterilization
*Death
Bacillus subtilis var. niger

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